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Distinct Behavior of Cardiac Myosin Heavy Chain Gene Constructs In Vivo

Discordance With In Vitro Results

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Transcriptional thyroid hormone responsiveness of the cardiac α-myosin heavy chain (α-MHC) gene has been demonstrated in transfections into fetal and neonatal cardiomyocytes and in transgenic mice. However, the correspondence between the regulation of MHC expression in dissociated cells with that in the intact heart is unclear. Given the cost and time involved in generating multiple transgenic lines for the characterization of gene regulatory elements, we used direct cardiac gene transfer to identify elements regulating both basal and thyroid hormone responsive cardiac α-MHC gene expression in the adult heart in vivo. Sequences upstream of the rat α-MHC gene linked to a luciferase reporter gene were injected into the hearts of adult rats subjected to various thyroid manipulations. The 161-bp sequence upstream of the transcription start site, which contains a TATA box, a CCAATT box, and a thyroid hormone response element, was transcriptionally active but not thyroid hormone responsive. The expression of a construct containing 388 bp of upstream sequence was increased by thyroid hormone administration, a response that required an intact thyroid hormone response element. However, expression of this construct failed to decrease to basal levels in a hypothyroid state. To confer complete (positive and negative) thyroid hormone regulation, 2,936 bp of upstream sequence was sufficient. These results demonstrate that, although necessary, the thyroid hormone response element is not sufficient for complete thyroid hormone regulation of this gene in vivo. In addition, DNA sequences regulating the quantitative expression of cardiac α-MHC in the euthyroid state have been demonstrated. One sequence, an MEF-2 site, which has been shown to be essential for high levels of expression of at least one other cardiac gene in neonatal cardiocytes, was mutated and found not to affect α-MHC expression in the adult heart. These data emphasize the complexity of gene regulation in an intact organ, aspects of which cannot be simulated in culture. (*Circulation Research* 1993;72:1211–1217)

KEY WORDS • cardiac α-myosin heavy chain • thyroid hormone • thyroid hormone response element

The adult mammalian heart is able to alter its patterns of gene expression in response to a diversity of hemodynamic and hormonal stimuli (see Reference 1). One adaptation that has been studied at a molecular level is the regulation of cardiac myosin heavy chain (MHC) isoform expression by thyroid hormone. The cardiac MHC isoforms are the products of two largely homologous genes, α and β, which are located in tandem and separated by ≈4.5 kb of intergenic sequence.[2,3] In the ventricular tissue of rodents and other small mammals, the amount of the αα-MHC homodimer increases, whereas that of the ββ-homodimer decreases in response to thyroid hormone. In contrast, the amount of the ββ-homodimer increases and that of the αα-homodimer decreases in hypothyroid states.[1,4-7] In vitro, thyroid hormone regulation of rat α-MHC gene expression has been shown to be mediated by the direct interaction of a thyroid hormone receptor with a thyroid hormone response element (TRE) in the 5′ flanking region of the α-MHC gene.[8-10] This sequence, located between −156 and −136 in the rat α-MHC gene, consists of two direct repeats separated by a spacer of four nucleotides and has been shown to bind the thyroid hormone receptor, resulting in increased α-MHC gene transcription.[8-11]

Studies delineating the molecular mechanism of thyroid hormone regulation of cardiac α-MHC gene expression have been carried out in dissociated myocytes derived from fetal hearts.[12,13] We were interested in determining whether the regulatory elements mapped in vitro would behave similarly in the adult heart in vivo. Fetal myocytes predominantly express the fetal ββ-MHC isoform, whereas the adult rat heart predominantly expresses the αα-MHC isoform.[7,14] It is generally assumed that interactions between multiple cis-acting sequences and trans-acting effectors are responsible for the transcriptional regulation of genes. It is not possible to model in vitro the complex hemodynamic and neurohumoral stimuli that are associated with a functional

cardiac system. Transgenic mice bearing the entire intergenic region between the mouse β-MHC (located 5' to α) and α-MHC genes linked to the chloramphenicol acetyltransferase (CAT) reporter displayed appropriate tissue and developmental regulation.[15,16] However, CAT activity was not present in any tissue from mice carrying a CAT transgene driven by =138 bp of upstream α-MHC sequence.[16]

In addition to thyroid hormone regulation, other sequences that modulate the expression of cardiac genes have been identified, including MCAT,[17-21] CArG,[22,23] and MEF-2.[24,25] One such sequence, termed HF-1, is a 28 bp element upstream of the rat myosin light chain-2 gene, which confers cardiac muscle-specific expression to a reporter gene. HF-1 contains homology to the E-box, CArG/serum response element, AP-2, and MEF-2 sites. Mutations in the MEF-2 site significantly reduce reporter gene expression when transfected into neonatal cardiac myocytes.[25]

We[26,27] and others[28,29] have recently reported a method of direct gene transfer into adult mammalian heart that allows the investigation of gene regulation in vivo. In the present study, we injected gene constructs containing various 5' flanking sequences (from −161 up to −2,936) of the rat α-MHC gene coupled to the firefly luciferase (luc) gene into the hearts of adult rats subjected to various thyroid hormone manipulations. Our data, although confirming the thyroid hormone responsiveness conferred by upstream sequences of α-MHC gene, differ from the results of in vitro experiments in the following way. None of the rat α-MHC constructs transfected into neonatal cardiocytes was expressed in the absence of thyroid hormone,[13] whereas all of the rat α-MHC constructs that we studied in vivo were expressed in both the adult hypothyroid and euthyroid states. Further, constructs containing short upstream sequences were positively responsive to thyroid hormone, a response requiring an intact TRE. In contrast to the results of cell culture transfections, however, our data demonstrate that these constructs remained active in the absence of hormone. Only the longest (2,936-bp) upstream sequence showed both positive and negative thyroid hormone responsiveness. Thus, although necessary, the TRE is not sufficient for positive and negative thyroid hormone responsiveness in vivo. In addition, sequences have been identified that modulate α-MHC expression in the adult euthyroid heart. One potential candidate for positive regulation, an MEF-2 site, was found not to affect quantitative expression in the adult heart.

Materials and Methods

Plasmids

α-MHCluc constructs contained various portions of the rat α-MHC 5' flanking sequence cloned into pXP2, a promoterless luc vector.[30] The −2,936-bp construct consisting of the rat α-MHC gene from −2,936 to +420 (relative to the transcription start site) was excised from pSVOMCAT[12] (kindly provided by B. Markham, Medical College of Wisconsin, Milwaukee) and cloned as a HindIII fragment into pXP2. The −1,696-bp construct was created by cloning a Bgl II fragment from the −2,936-bp construct into pXP2. In the remainder of the constructs, rat α-MHC sequences terminate at +32 rel-

ative to the transcription start site. Construction of the −613-bp construct was described earlier (pα-MHCluc[27]). The −540-, −388-, −300-, and −161-bp fragments were generated from pα-MHCluc as BamHI–Bgl II fragments by polymerase chain reaction using a known sequence.[2] The 5' sense primers used were CGGGATCCGTTGTC-CCTGGGGAGCCAGCA, CGGGATCCGGCCCGTA-AGAGAAGGTGAC, CGGGATCCGGAGGTGGTG-TGAGACGGTCC, and CGGGATCCGCTGCTGTCC-TCCTGTCACC, respectively, and the 3' antisense primer was GGAGATCTGCTAAACTCCTTCTTACTTGG-GAT. The −388δTRE construct, in which sites within the putative TRE (base pairs −142 and −143) were mutated from CC to TT, and the −388δMEF-2 construct, in which sites within the MEF-2 site (base pairs −330 and −331) were mutated from AA to CC, were generated from the −388 plasmid using the oligonucleotide method of site-directed mutagenesis.[31] Sequences were confirmed by DNA sequence analysis. pRSVCAT, in which the coding sequence of the CAT gene is spliced to long terminal repeat (LTR) sequences of the Rous sarcoma virus (RSV) has been previously described.[32] All plasmids were purified by alkaline lysis, CsCl gradient centrifugation, and dialysis and were stored in water at −20°C.

Animal Models

Adult female Wistar rats initially weighing between 175 and 200 g were used for all studies. To study thyroid hormone responsiveness, animals were made hypothyroid by administering propylthiouracil (PTU, 500 mg/l) in the drinking water for 3 weeks.[33] At this point, cardiac DNA injections were performed, and half of the animals were made hyperthyroid by daily injections of 3,5,3'-triiodothyronine (T₃, 200 μg/100 g i.p.); the remainder of the animals remained hypothyroid on PTU. Control euthyroid animals were maintained and injected in parallel. Animals were killed 5 days after cardiac injection. We have previously shown that this protocol results in marked shifts in endogenous MHC gene expression, to β predominance with PTU treatment and to α predominance with T₃ administration.[27] Four to eight animals subjected to each of the three thyroid hormone states were injected with each construct. Cardiac injection was performed as previously described.[26,27] Briefly, animals were anesthetized intraperitoneally with chloral hydrate (0.7–1.0 ml/100 g of a 4% solution), a left lateral thoracotomy was performed, the heart was briefly exteriorized, and injections were made directly into the apex of the left ventricle. The heart was then repositioned in the chest, the animal was briefly hyperventilated, and the incision was closed. Surgical and postoperative mortality was =10% in all groups. In the current study, 50 μl DNA solution containing 2.5 μg of the cellular promoter construct of interest and 1.5 μg pRSVCAT and 3% Evans blue dye (to confirm the intramuscular location of the injection) in 0.9% NaCl were injected through a 27-gauge needle. Because of concern that there might be competition among promoters for limiting transcription factors, data were also generated at a second DNA concentration by using 30 μg of some of the cellular promoter constructs with 5 μg pRSVCAT in a 50-μl injectate volume.

Reporter Gene Assays

Rats were killed 5 days after injection, and their hearts were removed. The atria and great vessels were trimmed, and the ventricles were washed in iced saline and weighed. The heart was then homogenized in 1 ml homogenization buffer without Triton X-100[34] with a Tissumizer (Tekmar Co., Cincinnati, Ohio). Homogenates were centrifuged at 6,000g for 30 minutes at 4°C, and then supernatants were frozen at −70°C for subsequent analysis. By use of a Monolight luminometer (model 2010, Analytical Luminescence Laboratory, San Diego, Calif.), luc activity was measured in 5% of the supernatant as previously described.[26,27] CAT activity was determined in 10% of the supernatant of samples in which 1.5 μg pRSVCAT was injected and in 5% of the supernatant in which 5 μg pRSVCAT was injected as previously reported.[26,27] Samples in which CAT conversion was less than 0.4% (background averaged 0.2%) were not reported. By this criterion, no more than two animals in any group were excluded, and no groups were selectively affected. All CAT conversions were in the linear range. Results are expressed as luc−background (in raw luminometer units)/CAT−background (as percent conversion).

Statistical Analysis

Results were subjected to a one-way analysis of variance (ANOVA) to establish differences between groups. The mean square error within groups was then used in Fisher's multiple comparison test to evaluate differences among groups. To correct for nonparametric differences among groups, the data generated with the −2,936-bp construct were subjected to a logarithmic transformation before ANOVA.[35] Significance, unless otherwise indicated, is reported at $p < 0.05$.

Results

The experimental design was to inject DNA constructs consisting of an α-MHC upstream sequence linked to a reporter gene into hearts of euthyroid animals or animals subjected to thyroid manipulation and to quantitate reporter gene activity 5 days later. This approach allowed for the identification of DNA sequences that mediate the positive and negative responsiveness of the α-MHC gene in vivo. To demonstrate the effectiveness of the hormonal treatments, heart and body weights of animals were determined. As expected, PTU treatment was associated with significant reductions in both heart and body weights versus the control condition. Heart and body weights were 0.50±0.01 and 214±5 g with PTU treatment versus 0.67±0.01 and 258±5 g in the control condition. Subsequent treatment with T_3 induced a 27% increase in heart weight and a 29% increase in the heart weight/body weight ratio relative to PTU-treated animals. The heart weight/body weight ratio was also increased in T_3-treated animals relative to control animals (3.3 versus 2.6 mg/g, $p < 0.01$). These characteristic changes coupled with the well-established changes in the relative levels of the endogenous α- and β-MHC mRNAs in similarly treated animals[27] are sufficient to establish the effectiveness of the hormonal manipulation.

In all animals, α-MHCluc constructs were coinjected with pRSVCAT, consisting of a constitutive viral promoter linked to the CAT gene. This control serves as an internal standard for gene transfer efficiency. This is of particular importance in the present study because of the discrepant sizes of the hearts being injected, which may influence the success of the injection, and also because the thyroid hormone status of the animal may theoretically influence the efficiency of gene transfer and/or RNA metabolism. CAT activity was equivalent in both PTU- and T_3-treated animals (5.2±1.7% versus 3.2±0.6%), ruling out a primary hormonal effect on the behavior of this construct. Both of these values were less than the values for euthyroid control animals (10.9±1.9%), which suggests that heart size at the time of gene injection may have influenced transfection efficiency. Similar CAT activities in hearts of PTU- and T_3-treated animals further suggest that changes in α-MHC promoter–linked reporter gene activities (luc) should reflect specific thyroid hormone effects.

The behavior of eight α-MHC promoter reporter gene constructs in adult euthyroid animals is shown in Figure 1. Since studies were repeated on several occasions, data are expressed as a percentage of the −388-bp construct, which was the most active on every occasion. Some of the lengths of upstream sequence were chosen to allow direct comparison with previous studies in cultured fetal cardiocytes.[13] All of the constructs (including the −161-bp construct) contain the TRE (located at position −156 to −136), TATA, and CAAT elements and are active in the normal adult heart. However, there are clear quantitative differences among the various constructs. First, as shown in Figure 1, the −161-bp construct contains enough information to be active in the adult heart. Second, there are positive regulatory element(s) located between −300 and −388 accounting for an approximate fourfold to fivefold difference in promoter activity between these constructs, and third, a negative regulatory element(s) is present between −613 and −1,696 that decreases promoter activity to lower levels than those obtained with the −161-bp construct. The 88-bp fragment between −300 and −388 contains a consensus MEF-2 site and binds MEF-2 fusion protein with plausible affinity as shown by electrophoretic mobility shift assay (E. Olson, M.D. Anderson Cancer Center, University of Texas, Houston, personal communication). To further investigate this potential positive regulatory element, we mutated the MEF-2 site, which is located at −325 to −335 (−388δMEF-2). This same mutation, when introduced into an MEF-2 site in the myosin light chain-2 gene, resulted in a decrease in transcriptional activity in neonatal cardiocytes.[25] In contrast, the activity of the α-MHC MEF-2 mutant was similar to the wild-type construct.

To define the elements of the rat α-MHC gene that mediate its response to thyroid hormone, the chimeric reporter gene constructs described above were coinjected with pRSVCAT into the hearts of hypothyroid animals. Half of the animals remained on PTU, and half were then treated with T_3. The relative activities of the α-MHC constructs (normalized to pRSVCAT) in hypothyroid and hyperthyroid animals are shown in Figure 2. The −161-bp construct, which contains the TRE, did not display thyroid hormone responsiveness in vivo. T_3 increased activity of the −388-bp construct fourfold ($p < 0.05$) and the −2,936-bp construct 20-fold relative
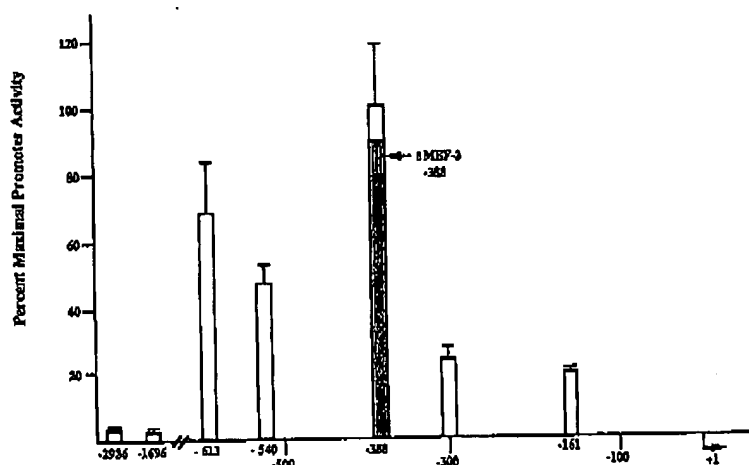
FIGURE 1.   *Bar graph showing the behavior of the α-myosin heavy chain constructs in euthyroid rat heart. The 5' flanking sequences of the α-myosin heavy chain gene (shown on the abscissa) linked to the firefly luciferase (luc) reporter gene were coinjected into rat heart with pRSVCAT, in which the coding sequence of the chloramphenicol acetyltransferase (CAT) gene is spliced to long terminal repeat sequences of the Rous sarcoma virus (RSV). Percent maximal promoter activities (luc/CAT) of each are shown on the ordinate and are expressed as a percentage of the −388-bp construct, which had maximal activity. Each bar represents the mean±SEM of six to nine studies. The δMEF-2 mutant (in which base pairs −330 and −331 were mutated from AA to CC, see text) is indicated in the shaded column and was not statistically distinct from the −388-bp construct.*

to the activity seen in the PTU group ($p<0.01$). Of note, the −161-, −388-, and −613-bp constructs showed significant transcriptional activity even in PTU-treated animals, in which endogenous α-MHC mRNA is nearly undetectable. Therefore, the activities of these constructs in adult hypothyroid animals is distinct from their activities in thyroid hormone-depleted fetal car-

diocyte cultures, in which transcriptional activity is barely detectable. Only the −2,936-bp construct was regulated in vivo in a manner that was concordant with the mRNA levels of the endogenous gene, increasing expression nearly sixfold in response to T_3 and showing a significant (3.4-fold) reduction in activity in PTU-treated animals relative to euthyroid control animals.
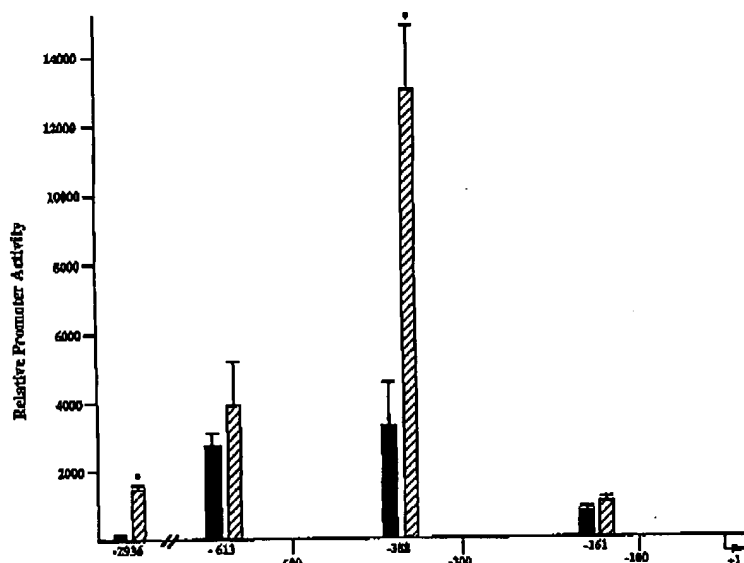

FIGURE 2.   *Bar graph showing thyroid hormone responsiveness of the α-myosin heavy chain constructs. The same constructs as in Figure 1 were injected into hearts of animals treated with propylthiouracil (filled bars) or animals subsequently treated with 3,5,3'-triiodothyronine (crosshatched bars). Data are mean±SEM of four to eight studies and are expressed as luciferase activity (in raw luminometer units) over chloramphenicol acetyltransferase activity (percent conversion). Propylthiouracil reduced the activity of the −2,936-bp construct to below basal levels. *3,5,3'-Triiodothyronine increased promoter activity of the −388-bp construct (p<0.05) and the −2,936-bp construct (p<0.01).*
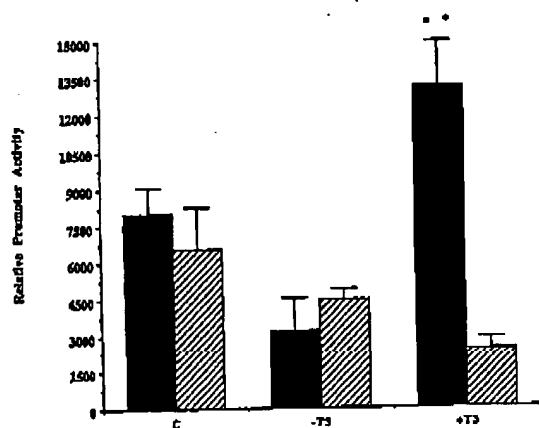
FIGURE 3. ·Bar graph showing the effect of the thyroid hormone response element (TRE) on α-myosin heavy chain (α-MHC) gene expression. T3, 3,5,3'-Triiodothyronine. Data are expressed as in Figure 2. Promoter activity was compared in two constructs: −388α-MHC–luciferase (filled bars) and −388δTRE (crosshatched bars), in which base pairs −142 and −143 were mutated from CC to TT (see "Materials and Methods") in three thyroid states. The mutation in the TRE did not effect promoter activity in euthyroid (control [C]) or hypothyroid (−T3) animals but abolished the T3-induced increase in promoter activity (+T3). *$p<0.05$ for −388α-MHC–luciferase in +T3 vs. −T3 states; +$p<0.05$ for −388α-MHC–luciferase vs. −388δTRE in +T3 state.

The data presented above were all generated using 2.5 µg cellular promoters and 1.5 µg pRSVCAT. Data have also been generated in all three groups of animals by using 30 µg of the −613-, −388-, and −161-bp constructs coinjected with 5 µg pRSVCAT, and the results with the higher dose of DNA were directionally identical: The −613-bp construct was 1.4 times more active, and the −388-bp construct was 1.9 times more active in T3-treated animals than in PTU-treated animals (both $p<0.05$). The behavior of the −161-bp construct was identical in PTU- and T3-treated animals and markedly reduced (≈10–15-fold, $p<0.01$) relative to the other two constructs. However, because it is possible for competition to occur between active promoters,[26] the figures reflect data obtained only with the smaller doses of DNA.

The data shown in Figure 2 demonstrate that the TRE is not sufficient to confer either positive or negative thyroid hormone responsiveness of the α-MHC promoter in vivo. To test the necessity of this TRE for positive thyroid hormone responsiveness, we mutated the TRE within the −388-bp construct: base pairs −142 and −143 were changed from CC to TT. This alteration has been shown to abolish T3 receptor binding and to ablate thyroid hormone responsiveness of the sequence in transfected CV-1 cells.[11] As shown in Figure 3, this mutation abolished the inducible expression of the construct by thyroid hormone but did not significantly alter its expression in euthyroid control animals or in PTU-treated animals. Thus, the TRE is required for positive thyroid hormone responsiveness.

Discussion

In the present study, we have used a technique of direct gene transfer into adult mammalian heart to characterize the activity of a cellular gene in the adult heart in vivo. The feasibility ;of the technique for mapping the regulatory elements of genes in cardiac tissue has been previously confirmed by the demonstration that cardiocytes are selectively transfected[28,29] and that cellular promoters behave in a tissue-restricted and thyroid hormone–responsive manner after in vivo gene transfer.[26,27] Potential limitations of the approach that can be controlled include the variability in transfection efficiency and the possibility that the two constructs injected may compete for limiting transcription factors. These issues are addressed by the current experiments. The issue of variability is largely resolved by normalization of the activity of the cellular promoter (α-MHC) to that of a constitutive promoter (RSV-LTR), which results in standard errors generally less than 20%. Competition for transcription factors is likely minimal at the doses of DNA injected, since directionally similar data are seen with DNA amounts that differ by 10-fold. In addition, we have shown that increasing amounts of pRSVCAT, up to 5 µg, injected into heart muscle in the presence of fixed amounts of pα-MHCluc does not reduce luc expression significantly (manuscript in preparation). Finally, it is unlikely that a subselective population of cells is targeted, since expression of the −2,936-bp construct parallels the steady-state levels of the endogenous α-MHC mRNA.

The general behavior of the 5' flanking sequence of the α-MHC gene in vivo (Figure 1) appears to reflect a complex array of cis-acting elements. Basal activity appears to be conferred within the initial 161 bases upstream from the transcription start site, which includes a TATA box, an MEF-1 site, and a CCAAT sequence in addition to the TRE.[2] Our data and data obtained from in vitro cell culture experiments both suggest the existence of positive regulatory element(s) between −300 and −388. The sequence between −300 and −388 includes an MEF-2 motif that has been shown to play a role in muscle-specific transcription. Also, this sequence contains an E-box, but it is not conserved between mouse and rat[2,15] and is therefore unlikely to be of functional significance. Mutation of the MEF-2 site in the rat myosin light chain-2 gene markedly reduces expression of a linked reporter gene in transfected cardiocytes.[25] In contrast, a similar mutation in the rat α-MHC MEF-2 sequence had no effect on expression in the adult heart in vivo. Future in vivo studies using either serial deletions or site-directed mutations should allow a more precise definition of the role of these and additional elements in cardiac gene regulation.

Our data differ significantly from the results of fetal cardiocyte transfection experiments[13] in several respects. First, our data demonstrate the presence of a strong negative element between −613 and −1,696 that was not observed in cell culture. Second, ur data do not demonstrate the existence of a negative regulatory element between −540 and −613 as suggested by the cell culture data.[13] Third, most of the constructs presented here have been tested and found to be inactive in

fetal or neonatal cardiocyte cultures grown in hormonally defined T_3-depleted serum, whereas they are all active in the adult hypothyroid and euthyroid heart. This suggests that the lack of expression in cultured cardiocytes may not merely be due to the absence of thyroid hormone.

In the adult rat heart, the endogenous α-MHC gene is exquisitely regulated by thyroid hormone; in the absence of T_3, little detectable mRNA is seen, and in the presence of T_3, mRNA levels increase dramatically.[3,5-7] Cell culture studies have identified a TRE located from −156 to −136 upstream from the α-MHC gene that binds the thyroid hormone receptor and results in increased rates of gene transcription.[8,10,11] Similar sequences have been identified in the regulatory regions of other thyroid hormone responsive genes, and these cis-acting sequences are members of a larger group of hormone responsive elements.[36,37] In primary neonatal cardiocytes, in which α-MHC gene expression is dormant, a minimal α-MHC promoter that includes the TRE and only 161 bases of 5' flanking sequence is sufficient to confer T_3 responsiveness to a reporter gene, although more dramatic hormone responsiveness is seen if longer stretches of upstream sequence are provided.[13]

Our data demonstrate that thyroid hormone regulation of the gene in vivo is more complex than previously appreciated. In contrast to the data generated in neonatal cardiocytes, the −161-bp construct is not thyroid hormone responsive in vivo. However, the presence of an intact TRE is necessary for thyroid hormone–inducible expression of the gene, since a mutation in the half site (Figure 3) abolishes T_3 responsiveness. Taken together, these two observations suggest that other cis-acting elements (present within the first 388 bases of upstream sequence), including a functional TRE, are involved in positive hormonal regulation in vivo.

In addition to these proximal regulatory elements, our data suggest that cis-acting sequences upstream of −613 are necessary for negative thyroid hormone responsiveness. These data are concordant with data generated by others,[15,16] who have shown that transgenic mice expressing the entire intergenic sequence upstream from the α-MHC gene coupled to CAT demonstrate appropriate positive and negative thyroid hormone regulation of the transgene but that mice expressing shorter constructs manifest significant CAT activity even when made hypothyroid (J. Robbins, University of Cincinnati [Ohio], personal communication).

In summary, the present study demonstrates both the feasibility and the usefulness of promoter mapping in vivo by use of the direct injection approach. The discordance between in vivo and in vitro data emphasizes the complexity of gene regulation in an intact organ, which likely involves interactions between multiple cis- and trans-acting elements responding to multiple influences, some of which cannot at present be modeled in culture.

Acknowledgment

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